

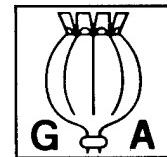
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13. ABSTRACT <i>(Maximum 200 words)</i> A rapid capillary electrophoresis (CE) method for the quantification for four biologically active biflavanones present in three different traditional "African medicinal preparations from the seeds of Garcinia kola was developed. The four biflavanones of interest (GB1, GB2 and GB1-glycoside and kolaviron) were quantified in a traditional tea preparation and two commercially available ethanolic formulations. The optimum separation conditions consisted of a 100 mM borate, pH 9.5 running buffer, which gave baseline resolution of all four components in less than 12 minutes. Linear calibration ranges for each component were between 2.5 and 1000 ug/ml. Limits of detection for the biflavanones quantified in this study were between 3 and 6 ug/ml. the "fingerprint" of the biflavanones in the aqueous tea and two ethanolic formulations was found to be similar, however concentrations of the four biflavanones were up to 50 fold higher in the ethanolic preparations. The major component in all three formulations was GB1.			
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Editor

Prof. Dr. Adolf Nahrstedt
Institut für Pharmazeutische Biologie
und Phytochemie der Universität
Hittorfstraße 56
D-48149 Münster
Germany
Fax: +49 251 83-383 41
E-mail: nahrste@uni-muenster.de

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raumacline (**2**) seems to have been performed already during the 1950 s but at the time a complete structure determination was not attainable [18]. The search for the appropriate enzymes for the raumacline (**2**) biosynthesis from ajmaline (**3**) pointed to a rather specific process catalyzed by membrane-bound peroxidase and NADPH-dependent reductase which reduced an opened dialdehyde intermediate of ajmaline (**3**) conversion [5], [19] (Fig. 2a). Deducing the biosynthetical pathway of the new compound **1** from vomilenine (**4**), we can suppose the formation of a structurally similar dialdehyde intermediate after deacetylation of **4** causing destabilization of the C-17 hydroxylated indole-nine skeleton followed by cleavage of the C7-C17 bond. Further reduction of a ring-opened (*chano*) form of the formed sarpagan alkaloid would result in formation of the putative deoxy-derivative of **1** (Fig. 2b). The hydroxylation of the indole moiety may be a late step of this biosynthetic pathway. However, final studies on the appropriate enzymes involved in the biosynthesis of **1** must be performed to clarify the biosynthetic process in detail.

The isolation of the new monoterpenoid indole alkaloid **1** from *R. serpentina* hairy root culture indicates its high potential in the production of new putatively pharmacologically-active substances. In addition, the here described *in vitro* plant system might be an important source for rare alkaloids which can be used as substrates for the detection of novel enzymes involved in the biosynthesis of ajmaline structurally related compounds, including the rare raumacline alkaloids. Such alkaloids might also be of interest for *in vivo* NMR monitoring of alkaloid formation by *R. serpentina* cell suspension cultures. Examples of successful applications of the alkaloids isolated from hairy roots of *R. serpentina* were displayed in our recent publications [20], [21].

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References

- Gräther O, Schneider B. Progress in Botany. The metabolic diversity of plant, cell and tissue cultures. In: Esser K, Lüttge U, Kadereit JW, Beyschlag W, editors. Vol. 62 Berlin: Springer Verlag, 2001: 266–304
- Falkenhagen H, Stöckigt J, Kuzovkina IN, Alterman IE, Kolshorn H. Indole alkaloids from hairy roots of *Rauwolfia serpentina*. Can. J. Chem. 1993; 71: 1–3
- Kuroyanagi M, Arakawa T, Mikami Y, Yoshida K, Kawahar N, Hayashi T, Ishimaru H. Phytoalexins from hairy roots of *Hyoscyamus albus* treated with methyl jasmonate. J. Nat. Prod. 1998; 61: 1516–9
- Zarate R. Tropane alkaloid production by *Agrobacterium rhizogenes* transformed hairy root cultures of *Atropa baetica* Willk. (Solanaceae). Plant Cell Rep. 1999; 18: 418–23
- Stöckigt J. The Alkaloids. Biosynthesis in *Rauwolfia serpentina*. Modern aspects of an old medicinal plant. In: Cordell GA, editor. Vol. 47 New York: Academic Press, 1995: 115–72 (and literature cited therein)
- Polz L, Stöckigt J, Takayama H, Ushida N, Aimi N, Sakai S-I. Biotransformation of ajmaline in plant cell cultures of *Rauwolfia serpentina* Benth.: the new indole alkaloids raumacline and *N*(β)-methyl-raumacline. Tetrahedron Letters 1990; 31: 6693–6
- Endreß S, Takayama H, Suda S, Kitajima M, Aimi N, Sakai S-I, Stöckigt J. Alkaloids from *Rauwolfia serpentina* cell cultures treated with ajmaline. Phytochemistry 1993; 32: 725–30
- Court WE. Alkaloid distribution in some African *Rauvolfia* species. Planta Medica 1983; 48: 228–33
- Nasser AMG, Court WE. Stem bark alkaloids of *Rauvolfia caffra*. J. Ethnopharmacol. 1984; 11: 99–117
- Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 1962; 5: 473–97
- Smith JI, Smart NJ, Kurz WGW, Misawa M. The use of organic and inorganic compounds to increase the accumulation of indole alkaloids in *Catharanthus roseus* (L.) G. Don cell suspension cultures. J. Exp. Bot. 1987; 38: 1501–6
- Shelud'ko YV, Kostenyuk IA. Growth dynamics and total alkaloid content in the transgenic root culture of *Rauwolfia serpentina* Benth. Tsitologiya i Genetika (Rus.) 1994; 28: 35–8
- Sheludko Y, Gerasimenko I, Stöckigt J. *Rauvolfia serpentina* hairy root cultures as a source of new indole alkaloids of the sarpagine group. In: Natural Products Research in the New Millennium, 48th Annual Congress of the Society for Medicinal Plant Research. Zurich, Switzerland. September 3–7 2000. Abstracts Zurich: 2000: P1A/36
- PCMODEL v. 7.0. Serena Software. Bloomington, 1999
- Stöckigt J, Pfitzner A, Firl J. Indole alkaloids from cell suspension cultures of *Rauwolfia serpentina* Benth. Plant Cell Rep. 1981; 1: 36–9
- Warzecha H, Obitz P, Stöckigt J. Purification, partial amino acid sequence and structure of the product of raucaffricine-O- β -D-glucosidase from plant cell cultures of *Rauwolfia serpentina*. Phytochemistry 1999; 50: 1099–109
- Lounasmaa M, Hanhinen P. Interpretation of the formation of nine “artifact indole alkaloids” in the ajmaline/sarpagine series. J. Nat. Prod. 2000; 63: 1456–60
- Gorman M, Neuss N, Djerassi C, Kutney JP, Scheuer PJ. Alkaloids of some Hawaiian *Rauvolfia* species: the structure of sandwicine and its interconversion with ajmaline and ajmalidine. Tetrahedron 1957; 1: 328–37
- Obitz P, Endress S, Stöckigt J. Enzymatic biosynthesis of raumacline. Phytochemistry 1995; 40: 1407–17
- Falkenhagen H, Stöckigt J. Enzymatic biosynthesis of vomilenine, a key intermediate of the ajmaline pathway, catalyzed by a novel cytochrome P 450 dependent enzyme from plant cell cultures of *Rauwolfia serpentina*. Z. Naturforsch. 1995; 50C: 45–53
- Hinse C, Sheludko Y, Provenzani A, Stöckigt J. *In vivo* NMR at 800 MHz to monitor alkaloid metabolism in plant cell cultures without tracer labeling. J. Am. Chem. Soc. 2001; 123: 5118–9

Capillary Electrophoresis Determination of Biflavanones from *Garcinia kola* in Three Traditional African Medicinal Formulations

Chris O. Okunji^{1,2}
Tantalia A. Ware²
Rickey P. Hicks²
Maurice M. Iwu^{1,2}
David J. Skanchy²

Abstract

A rapid capillary electrophoresis (CE) method for the quantification of four biologically active biflavanones present in three different traditional African medicinal preparations from the seeds of *Garcinia kola* was developed. The four biflavanones of interest (GB1, GB2 and GB1-glycoside and kolavironone) were quantified in a traditional tea preparation, and two commercially available ethanolic formulations. The optimum separation conditions consisted of a 100 mM borate, pH 9.5 running buffer, which gave baseline resolution of all four components in less than 12 min-

utes. Linear calibration ranges for each component were between 2.5 and 1000 µg/mL. Limits of detection for the biflavanones quantified in this study were between 3 and 6 µg/mL. The "fingerprint" of the biflavanones in the aqueous tea and two ethanolic formulations was found to be similar, however concentrations of the four biflavanones were up to 50 fold higher in the ethanolic preparations. The major component in all three formulations was GB1.

Key words

Capillary electrophoresis · *Garcinia kola* · kolaviron · biflavanone

Introduction

The seeds of *Garcinia kola* enjoy a folk reputation in African traditional medicine, and they have been used in many herbal preparations either singly or in combination with other plants. Among these are a tea prepared from a blend of *G. kola* and *Combretum micratum* and two ethanolic formulations consisting of blends of kolaviron and other natural products. *G. kola* contains a complex mixture of biflavonoids [1], [2], [3], prenylated benzophenones [4] and xanthone [5]. Kolaviron, is a mixture of C-3/C-8 linked biflavonoids found in *G. kola* and has been shown to have hepato-protective activity [6], [7], bronchodilator effect [8], and antidiabetic activity [9]. A number of products derived from *G. kola* have been manufactured and marketed as dietary supplements or phytomedicines. Like many dietary supplements, *Garcinia* products are susceptible to chemical variability

due to growth, harvest, drying, and storage conditions. Therefore suitable analytical methods are needed for the growing demand of dietary supplements including those containing *G. kola* to ensure their quality, safety and efficacy.

Recently, capillary electrophoresis (CE) has proven to be a useful technique for the separation of a variety of natural products, including flavanoids and their glycosides from various sources [10], [11], [12], [13], [14], [15], [16], [17]. The use of buffers containing components which can interact with the analytes to form complexes such borate [11], [12], [13] and micellar forming surfactants such as SDS [13], [15] and cetyltrimethylammonium bromide (CTAB) [16] have been used to enhance selectivity and provide an electrophoretic separation mechanism when the analytes do not bear a native charge. CE methods for the analysis of the constituents in traditional Chinese herbal remedies have re-

Affiliation

¹ International Centre for Ethnomedicine and Drug Development, Nsukka Nigeria; and Bioresources Development & Conservation Programme Silver Spring, MD, USA

² Department of Medicinal Chemistry, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Silver Spring, MD, USA

Correspondence

CPT David J Skanchy · Forensic Toxicology Drug Testing Laboratory · Attn: MCHL-UDL · 2490 Wilson Street · Fort George G. Meade, MD 20755-5235, USA · Phone: +1 301-677-3755 · Fax: +1 301-677-3714 · E-Mail: skanchyd@emh1.ftmeade.army.mil and dskanchy@yahoo.com

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Bibliography

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cently been reported [14] and are efficient methods for both fingerprinting and quantifying components of these herbal preparations. In this work we report a CE method for the quantitative analysis of biologically active biflavanones present in three traditional African medicinal preparations from the seeds of *Garcinia kola*.

Materials and Methods

Chemicals

Ethanol and d_6 -acetone were purchased from Aldrich (Milwaukee, WI, USA). Sodium hydroxide was purchased from Hewlett Packard (Waldbronn, Germany). Boric acid was obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Healers' Botanika™ Hangover Tonic (tonic) and Healers' Botanika™ Streptol (strep-tol) was purchased from Axxon Biopharm™ (Silver Spring, MD, USA). Water was purified with a Millipore™ system (Milford, MA). Tea bags were prepared by enclosing about 2 grams of ground *Garcinia kola* seed in a sachet.

Seeds of *G. kola* were brought at a local market at Orba Nsukka, Enugu State, Nigeria in October 1999 and the sample was authenticated by Dr. J.C. Okafor of the Forestry Department Enugu, Nigeria. Voucher specimens ICEDD991002, have been deposited in the Department of Pharmacognosy, the University of Nigeria, and Bioresources Development and Conservation Programme (BDCP) Herbaria. The reference standards used for the quantitative determination were purified in our laboratory from kola-viron extract of *G. kola* seeds according to the technique reported by Iwu and Igboho [3].

Working up the organic extract from 10.0 g of *G. kola* yielded four major compounds in sufficient amounts and purity (>98%) to be used as standards. Purity of the isolated standards was determined from HPLC and CE analysis of each purified compound. The compounds were identified by comparison of their spectral data and R_f values with those of the authentic isolates as 3",3",4",5",7,7"-heptahydroxy-4"-methoxy-3,8"-biflavanone (kolaflavonone); 3",4",4",5,5",7,7"-heptahydroxy-3,8"-biflavanone (GB1); 7"-O-alpha-D-glucopyranosyloxy-3",4",4",5,5",7-hexahydroxy-3,8"-biflavanone. (GB1 glucoside) and 3",3",4",4",5,5",7,7"-octahydroxy-3,8"-biflavanone (GB2). The structures of these compounds are shown in Fig. 1 showing relative configurations based on NMR data.

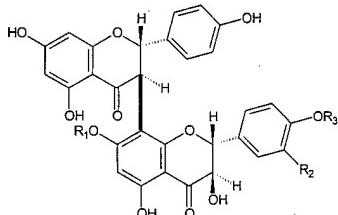


Fig. 1 Structures of biflavanones from *Garcinia kola*.

	R ₁	R ₂	R ₃
Kolaflavonone	H	H	CH ₃
GB1	H	H	H
GB1-7"-O-glucoside	Glc	H	H
GB2	H	OH	H

Instrumentation

All CE experiments were performed on a Hewlett Packard 3DCE System (Hewlett Packard, Waldbronn, Germany). All separations were performed in a fused-silica capillary (Polymicro Technology, Phoenix AZ, USA) having a total length of 63.5 cm with an effective length of 55 cm and an internal diameter of 50 μm at a constant temperature of 25 °C. The applied voltage was 28.5 kV and the current was 45 μA . Samples were injected hydrodynamically with 50 millibar of positive pressure applied for 2 seconds. Mesityl oxide or a system peak was used to measure electroosmotic flow (EOF). UV spectra were obtained from 200–600 nm for each component with the CE diode array detector, quantification was performed at a wavelength of 325 nm.

All ¹H-NMR data were collected using a Bruker DRX-600 spectrometer. Acetone- d_6 was used as the solvent for the standard sample. The degraded sample was dissolved in CE background electrolyte with the addition of 10% D₂O. The two-dimensional WATERGATE-TOCSY experiment employed a modified MLEV-17 (18–20) spin-lock sequence for a total mixing time of 80 ms, including the 2.5 ms trim pulses at the beginning and the end of the spin-lock sequence.

Procedures

The experimental details concerning the extraction and isolation procedures of *G. kola* biflavanoids have been described [3], [21]. Identity of the purified fractions was confirmed by TLC and mass spectrometric analysis. Standard solutions at a concentration of 1 mg/mL of GB1, GB1-glucoside, GB2, and kolaflavone (KF) were prepared by dissolving the pure components in 50/50 ethanol and water. The standard solutions were ultrasonicated for 5 minutes prior to serial dilution. Tonic and streptol samples were diluted 10 fold and 20 fold, respectively, with 50/50 ethanol and water prior to analysis in order to bring the most abundant component within the calibration range. A tea bag containing 2.15 g of ground *Garcinia kola* seed was placed into 110 mL of water at 90 °C. The tea was then steep covered for 10 minutes. The tea bag was squeezed gently and removed. The tea was centrifuged at 10,000 rpm for 5 minutes to remove particulates. Recovery of biflavanoids from the aqueous extraction was estimated to be about 4.9% of the total present in the seeds. All solutions were stable for several days, but were prepared fresh daily.

Results and Discussion

Analytical conditions

Borate buffers at high pH have been shown to be superior for the separation of mono-flavones and their glycosides [11], [12]. The high pH is necessary to ionize the acidic phenolic groups, and the ability of borate to complex with diols on both the aglycone and the attached sugars in the glycosides can further enhance resolution through differential complexation. Optimum conditions for the separation of the four major components quantified in this study and eleven other unidentified components present in the herbal preparations were 100 mM borate buffer at pH 9.5. A representative electropherogram of the tonic run under these conditions is shown in Fig. 2. Under these conditions all of the compounds had a negative charge and the elution order of the major components (KF, GB1, GB1-glucoside, and GB2) can be explained by the ability of these components to complex with the

borate buffer. Kolaflavanone lacks the diol needed for effective complexation and elutes first due to the lower negative charge density. The diol at the 3'' and 4'' position in GB 2 allows for strong borate complexation resulting in a longer elution time. Since GB1 and GB1-glucoside both lack this diol, they also have decreased ability to complex with borate and exhibit lower elution times compared to GB2. The elution of GB1-glucoside after both KF and GB1 is likely due to borate complexation with diols present in the sugar moiety. Although *cis*-1,2-diols are optimum for the formation of borate sugar complexes [12], the glucoside must have some ability to form a complex based on its elution after GB1. At pH 9.5 ionization of sugar hydroxy groups would not be a factor affecting the electrophoretic mobility.

The CE assay was very reproducible in terms of analysis times as long as a simple rinsing procedure was followed. Table 1 summarizes the reproducibility of the analysis times for each component for nine successive injections. Relative standard deviations for the migration times were about 1%. When a relative migra-

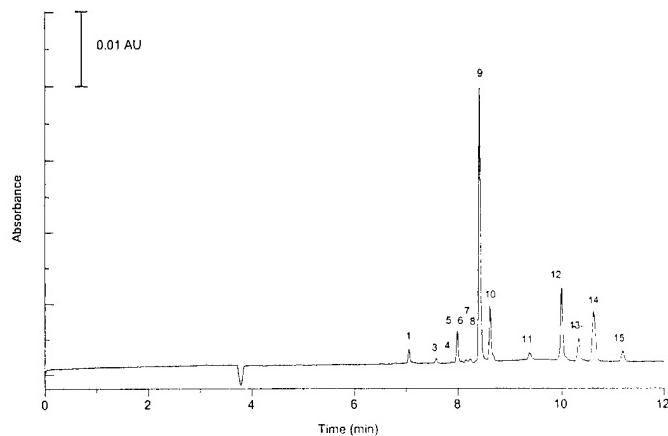


Fig. 2 Electropherogram showing the separation of biflavanone components in the tonic preparation. Run conditions: 100 mM borate buffer pH 9.5, 100 mBar sec injection, $E = 447 \text{ V/cm}$, $\lambda = 325 \text{ nm}$. See Table 4 for peak identification.

Table 1 Reproducibility of analysis times for biflavanoids ($n = 9$)

Analytes	Avg. μ_{ep} ($\text{cm}^2/\text{V sec}$) $\times 10^4$	Avg. migration Time (min) (S.D.)	% RSD	Avg. rel. migration time (min) (S.D.)	% RSD
KF	-2.85	8.13 (0.08)	0.96	2.13 (0.01)	0.51
GB1	-2.98	8.57 (0.09)	1.00	2.25 (0.01)	0.58
GB1-g	-3.04	8.78 (0.09)	1.01	2.31 (0.01)	0.56
GB2	-3.37	10.24 (0.11)	1.10	2.69 (0.01)	0.43

Table 2 Linearity and Assay parameters for the four biflavanoids

Analytes	Concentration range ($\mu\text{g/mL}$)	Slope	Intercept	R^2	LOQ ($S/N = 5$) ($\mu\text{g/mL}$)	LOD ($S/N = 3$) ($\mu\text{g/mL}$)
KF	2.5–1000	0.029	0.132	0.9998	5.16	3.10
GB1	2.5–1000	0.042	-0.121	0.9998	3.16	1.90
GB1-g	2.5–1000	0.042	-0.086	0.9999	4.18	2.51
GB2	2.5–1000	0.039	-0.185	0.9997	4.59	2.76

tion time (ratio of analyte migration time to neutral marker migration time) was used to correct for slight differences in electroosmotic flow the RSD fell to about 0.5 %. These numbers are comparable to other CE methods for flavonoids reported in the literature [14].

Linearity and limits of detection

Table 2 summarizes the calibration data and other assay parameters obtained for the four biflavanones. Corrected peak areas (peak area/ analysis time) were used in all quantitative analysis. Calibration curves were linear over the 2.5–1000 $\mu\text{g/mL}$ concentration range examined. Limits of detection, defined as a signal to noise ratio of three, ranged from 1.9 to 3.1 $\mu\text{g/mL}$. Limits of quantitation, defined as a signal to noise ratio of five, ranged from 3.16 to 5.16 $\mu\text{g/mL}$.

Quantitative and qualitative determination of biflavanones in herbal preparations

Table 3 summarizes the quantitative data obtained for the four biologically active biflavanones. The streptol preparation contained the highest levels of all four biflavanoids, with GB1 and GB2 the major components. The tonic contained slightly lower levels of these components, but the relative concentrations were similar. Clearly, the organic content (40% ethanol) in the two commercial tinctures significantly enhances the levels of all the biflavanoids compared to the aqueous tea preparation.

Concentrations of the major components are at least a 100-fold greater in the ethanolic formulations. Levels of the major components followed the same relative trend in the tea, with GB1 and GB2 the major components.

In addition to the four major components quantified in this study, several other unidentified components were resolved under optimum conditions, providing a detailed "fingerprint" of each formulation. A comparison of the relative analysis times of the minor peaks found in all three preparations revealed that 15 unique components (including the four major components quan-

tified) were resolved in the assay. These components are listed in Table 4, along with the percent of the total corrected peak area for each component found in each of the three formulations. Although no quantitative information is available for the minor components resolved in this study due to a lack of suitable standards, a comparison of the peak profiles and relative area percents should be useful for "fingerprinting" each of the preparations and for assessing quality and batch reproducibility of the herbal products. The assay could also prove useful in component analysis of crude extracts of the plant materials to assess quality prior to formulation. Previously, others have shown CE methods for flavonoids to be useful in analyzing levels of flavones in crude extracts from plants and to determine concentrations of specific components as well as for general fingerprinting of the plant species for phytotaxonomic comparisons [22]. This assay has already proven useful in our lab for the qualitative analysis of crude extracts for following the fractionation and purification process used to isolate standards of the major components.

Isomerization of GB1-glucoside in borate buffer

In the course of developing the method some samples were diluted in running buffer prior to analysis. Interestingly, in the case of GB1-glucoside, a noticeable shoulder appears on the peak after one hour in the borate buffer, indicating degradation to a second component. After 18 hours at room temperature in borate run-

ning buffer (100 mM, pH 9.5) the ratio of the two components was nearly equal (Fig. 3). No stability problem was observed in either ethanol : water or in a non-complexing buffer such as phosphate at a similar pH and concentration (data not shown). Because borate buffers are reported in the literature for the separations of flavonoid glycosides, we undertook a series of NMR experiments to elucidate the structure of this degradation product. Table 5 summarizes the results of NMR experiments on both GB1-glucoside standard in d_6 -acetone and a degraded sample left in borate buffer for over 24 hours. The stereochemistry of the anomeric protons of the glucosides of GB1 were assigned by analogy to the $^1H/^2H$ coupling constants and chemical shifts of known glucoside systems [23], [24]. The α -stereochemistry yields a coupling constant in the range between 3 and 5 Hz while the β -stereochemistry yields a coupling constant about 8 Hz. Overall, the NMR data strongly suggest a mixture of the α and β forms of the glycoside are present after treatment with buffer. The β epimer has a slightly longer elution time and probably forms a more stable complex with the borate background electrolyte than the α epimer resulting in partial resolution of the two peaks in the CE assay. The lack of isomerization observed in non-complexing buffer suggests that an interaction with borate may catalyze the conversion from the α to β forms.

Conclusions

This study has successfully demonstrated that CE can serve as a direct, robust and rapid method for both the quantitative and qualitative analysis of the biflavonoid constituents in different herbal formulations derived from *G. kola*. The distribution of the biflavonoids (GB1, GB2, GB1-glucoside and KF) in the three formulations was found to be qualitatively and quantitatively different. The commercially formulated tinctures contained more components and much higher concentrations of the four biflavonones quantified in this study. The unique and powerful capabilities of CE including high resolution and short analysis times, make it a powerful analytical tool in the quality control of these herbal products and other flavonoid-containing products such as *Ginkgo biloba* proprietary products.

Table 3 Levels ($\mu\text{g/mL}$) of biflavonones found in three different herbal preparations ($n = 3$)

Biflavonone	Tea	Streptol	Tonic
	Concentration ($\mu\text{g/mL}$) (S.D.)/% RSD		
KF	5.06 (0.14)/2.77	861 (48)/5.57	582 (14)/2.41
GB1	58.84 (0.86)/1.46	5593 (91)/1.63	3688 (79)/2.14
GB1-glucoside	6.46 (0.22)/3.41	820 (28)/3.41	735 (7.8)/1.06
GB2	29.68 (0.36)/1.21	1935 (40)/2.07	1165 (65)/5.58

Table 4 Component analysis (% total peak area) of herbal preparations (u = unidentified component, * = not detected)

Peak/identity	Migration time (min)	Tea	Streptol	Tonic
1/u	7.06	*	*	2.64
2/u	7.40	1.87	*	*
3/u	7.58	*	*	0.94
4/u	7.89	2.58	0.42	0.53
5/KF	7.99	7.05	6.60	5.67
6/u	8.06	*	0.36	0.48
7/u	8.15	*	0.25	0.78
8/u	8.23	*	1.04	0.87
9/GB1	8.41	57.57	55.45	46.10
10/GB1-g	8.61	5.12	9.27	10.05
11/u	9.38	*	0.95	1.60
12/GB2	10.00	23.23	16.25	12.29
13/u	10.33	2.58	3.92	4.51
14/u	10.63	*	4.74	11.52
15/u	11.20	*	0.75	2.00

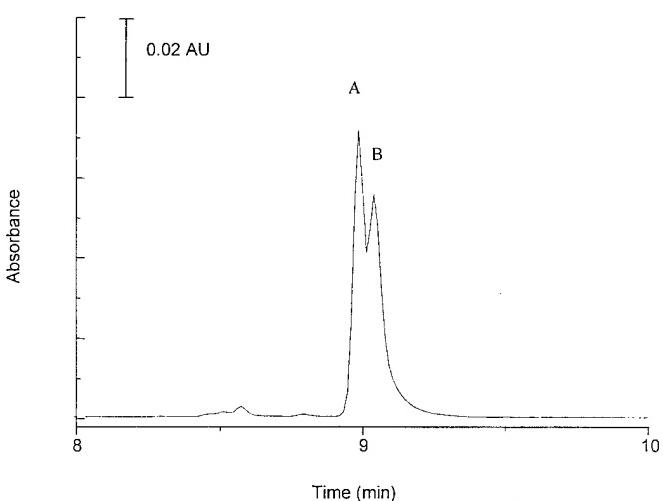


Fig. 3 Electropherogram of GB1-glucoside, α -epimer (A) dissolved in 100 mM, pH 9.5 borate after 18 hrs at room temperature. Electropherogram shows the formation of the β -epimer (B). Run conditions as in Fig. 2.

Table 5 Summary of the chemical shift and coupling constant data for glucosyl protons showing α to β isomerization

Compound	Chemical shift for anomeric proton	Stereochemistry coupling constant	Chemical shifts of remaining protons
GB1-glucoside in acetone	5.60 ppm	α 5 Hz	2.94, 2.74, 2.70 ppm
GB1-glucoside (borate buffer) ^a	5.34 ppm	α 4Hz	2.91, 2.87, 2.65 ppm
GB1-glucoside (borate buffer) ^a	5.19 ppm	β > 7Hz	4.28, 3.09, 3.04, 2.75, 2.72 ppm

References

- ¹ Terashima K, Aqil M and Niwa M. Garcinianin, A. Novel biflavonoids from the roots of *Garcinia kola*. *Heterocycles* 1995; 41: 2245–50
- ² Terashima K, Kondo Y, Aqil M, Waziri M, Niwa M. A study of biflavonones from the stem of *Garcinia kola* (Guttiferae). *Heterocycles* 1999; 50: 238–90
- ³ Iwu MM, Igboko O. Flavonoids of *Garcinia kola* seeds. *Journal of Natural products*. Llyodia 1982; 45: 650–1
- ⁴ Hussain RA, Owegby AG, Parimoo P, Waterman PG. Kolanone, a novel polyisoprenylated benzophenone with antimicrobial properties from the fruit of *Garcinia kola*. *Planta Med* 1982; 44: 78–81
- ⁵ Iwu MM. Handbook of African Medicinal Plants. Pub, CRC Press, Florida: p. 184 1993
- ⁶ Iwu MM, Igboko OA, Onwuchekwa UA, Okunji CO. Evaluation of the antihepatotoxic activity of the biflavonoids of *Garcinia kola* seed. *J. Ethnopharm.* 1987; 21: 127–38
- ⁷ Nwankwo JO, Tahnteng JG, Emerole GO. Inhibition of aflatoxin B1 genotoxicity in human liver-derived HepG2 cells by kolaviron biflavonoids and molecular mechanisms of action. *Eur. J. Canc. Prev.* 2000; 9: 351–61
- ⁸ Orié NN, Ekon EU. The bronchodilator effect of *Garcinia kola*. *East Afr Med J.* 1993; 70: 143–5
- ⁹ Iwu MM, Igboko OA, Okunji CO, Tempesta MS. Antidiabetic and aldose reductase activities of biflavanones of *Garcinia kola*. *J. Pharm. Pharmacol.* 1990; 42: 290–2
- ¹⁰ Issaq HJ. Capillary electrophoresis of natural products-II. Electrophoresis. 1999; 20: 3190–202
- ¹¹ Morin P, Villard F, Dreux M. Borate complexation of flavonoid-O-glycosides in capillary electrophoresis, 1. Separation of flavonoid-7-O-glycosides differing in their flavonoid aglycone. *J. Chromatogr.* 1993; 628: 153–60
- ¹² Morin P, Villard F, Dreux M, Andre P. Borate complexation of flavonoid-O-glycosides in capillary electrophoresis, 2. Separation of flavonoid-3-O-glycosides differing in their sugar moiety. *J. Chromatogr.* 1993; 628: 161–9
- ¹³ Pietta P, Mauri P, Bruno A, Gardana C. Influence of structure on the behavior of flavonoids in capillary electrophoresis. *Electrophoresis* 1994; 15: 1326–31
- ¹⁴ Wang CY, Huang HY, Kuo KL, Hsieh YZ. Analysis of *Puerariae radix* and its medicinal preparations by capillary electrophoresis. *J. Chromatogr. A.* 1998; 802: 225–31
- ¹⁵ Pietta P, Mauri P, Bruno A, Zini L. High-performance liquid chromatography and micellar electrokinetic chromatography of flavonol glycosides from *Tilia*. *J. Chromatogr.* 1993; 638: 357–61
- ¹⁶ Bjergegaard C, Michaelsen S, Mortensen K, Sorensen H. Determination of flavonoids by micellar electrokinetic capillary chromatography. *J. Chromatogr. A.* 1993; 652: 477–85
- ¹⁷ Song JZ, Xu HX, Tian SJ, Bu PP. Determination of quinolizidine alkaloids in traditional Chinese herbal drugs by nonaqueous capillary electrophoresis. *J. Chromatogr. A.* 1999; 857: 303–11
- ¹⁸ Bax A, Davis DG. MLEV-17 based two-dimensional homonuclear magnetization transfer spectroscopy. *J. Magn. Reson.* 1985; 65: 355–60
- ¹⁹ Eich G, Bodenhausen G, Ernst RR. MLEV-17 coherence transfer by isotropic mixing: Application to proton correlation spectroscopy. *J. Am. Chem. Soc.* 1982; 104: 3731
- ²⁰ Sklenar V, Piotto M, Leppik R, Saudek V. Gradient-tailored water suppression for ¹H-¹⁵N HSQS experiments optimized to retain full sensitivity. *J. Magn. Reson. Series A.* 1982; 102: 241–5
- ²¹ Iwu MM. Antihepatotoxic constituents of *Garcinia kola* seeds. *Experientia* 1985; 41: 699–70
- ²² Liang HR, Siren H, Jyske P, Reikkola ML, Vuorela P, Vuorela H, Hiltunen R. Characterization of flavanoids in extracts from four species of *Epimedium* by micellar electrokinetic capillary chromatography with diode array detection. *J. Chromatogr. Sci.* 1997; 35: 117–25
- ²³ Homans SW. In: *NMR of Macromolecules A Practical Approach*. Roberts GCK. : pp. 289–313 1993 Oxford University Press
- ²⁴ Hicks RP, Sneden AT. Preparation of glucosidic derivatives of steganol. *J. Nat. Prod.* 1985; 48: 357–62